FLUORESCENCE POLARIZATION OF DYE-LABELED COLLAGEN: LOCAL FLEXIBILITY IN THE NONHELICAL REGION

D. QUIMBY* AND LEE P. WITNAUER

Eastern Regional Research Center†
Philadelphia, Pennsylvania 19118

ABSTRACT

The fluorescent dye derivatives fluorescein isothiocyanate (FI) and 5-dimethylamino-1-naphthalene sulfonyl chloride (DNS) have been covalently bound to collagen. Fluorescence from the FI conjugate is depolarized primarily by rotation of the dye molecule about the covalent bond joining it to the protein. DNS on the other hand binds tightly to the helical regions of collagen and fluorescence from the DNS conjugate is depolarized by rotatory diffusional motion in the nonhelical regions of the protein. Variation in pH between 3 and 10.5 has little effect on the degree of fluorescence polarization of the DNS conjugate, indicating that the degree of flexibility in the nonhelical regions remains unchanged in this pH range. Ca^{2+} ion induces local flexibility in the helical regions of collagen, and removal of the nonhelical regions by pronase treatment seems to render the helical regions more susceptible to perturbation by Ca^{2+} ions.

INTRODUCTION

Weber introduced the technique of studying the structure and behavior of proteins in solution by observing the polarization of fluorescence from small dye molecules covalently bound to the protein (1). This technique has been used to study a large variety of proteins but has not up until now been applied to the study of collagen. In this paper we report a fluorescence polarization study of collagen in solution.

The general structure of collagen is now well established (2). The triple-helical arrangement of peptide chains produces a highly asymmetric protein with an axial ratio of about 200 to one. At least two types of secondary structure occur in collagen. One is the helical structure of each peptide chain in the triple helix. This helical configuration is sterically allowed by the presence of a glycine residue at every third position along the chain. In the terminal regions of the

*Research performed under tenure of an NRC-ARS Postdoctoral Research Associateship.

†Agricultural Research Service, U. S. Department of Agriculture.

protein, glycine does not appear as every third residue and these regions have an unknown, presumably nonhelical, secondary structure. Removal of the teleopeptides by proteolytic cleavage with enzymes such as pronase or pepsin has been found to affect profoundly some properties, such as the degree of aggregation (3), while leaving others unaffected, such as the thermal transition temperature in acid and neutral solution (4). It has been established that covalent crosslinks form in the nonhelical regions (5). Beyond this little is known about the structure or flexibility of these regions. One of the dyes used in this study, dansyl chloride (DNS), binds to collagen in such a way as to allow study of the flexibility of the nonhelical regions of the protein.

EXPERIMENTAL

Materials

Calfskin corium collagen was obtained by methods previously reported (6). The collagen was purified by repeated (3X) precipitation with 15 percent KCl, followed by redissolution in 0.1 M acetic acid. Pronase was obtained from Calbiochem‡. The dyes, fluorescein isothiocyanate (FI) and 5-dimethylamino-1-naphthalene sulfonyl chloride (DNS), were used as 10 percent (w/w) dispersions on cellite (Calbiochem).

Dye Coupling

Collagen was precipitated from 0.1 M acetic acid with 15 percent KCl and taken up in five percent Na_2CO_3 (pH 10.5 at 10°C.). The solutions usually contained about 0.3 percent collagen. The dye-cellite was then added, and the mixture stirred for about two hours at 10°C. Typically, the molar ratio of dye collagen during coupling was 5/1 for FI and 40/1 for DNS. At the end of the coupling period, the mixture was centrifuged and the protein precipitated from the supernatant with KCl. The dye-protein conjugate was taken up in 0.1 M acetic acid, centrifuged, and precipitated with KCl. This process was repeated until the protein was free of unbound dye, as indicated by the absence of dye fluorescence from the supernatant. The degree of dye labeling was calculated, using molar extinction coefficients of 4.3×10^3 at 330 nm for DNS, 3.4×10^4 at 500 nm for FI, a molecular weight of 300,000 daltons for collagen, and a density of 1.0 for the collagen solutions. For conjugates prepared as described in the Experimental section, the average number of dye molecules per collagen molecule was 2.0 for DNS and 0.13 for FI.

Pronase Treatment

Collagen-dye conjugates were treated with pronase (one percent by weight) in 0.1 M calcium acetate (pH 7.0) (5). The mixtures were gently stirred at 20°C.

‡Reference to brand or firm name does not constitute endorsement by the U. S. Department of Agriculture over others of a similar nature not mentioned.

for 18 to 20 hours. After treatment, collagen was precipitated with KCl and then redissolved in 0.1 M acetic acid and reprecipitated twice more.

Equipment and Measurements

Excitation light was provided by a 150 watt Xe discharge lamp. Excitation and emission wavelengths were selected with individual monochromators (Schoeffel Instrument Corporation, GM 250). The excitation light was polarized with a calcite prism. The fluorescence was viewed at 90° to the exciting beam and the polarization analyzed with a polaroid film, HN32. The polarization was corrected for grating effects, as suggested by Chen and Bowman (7). FI fluorescence was excited at 450 nm and viewed at 550 nm. DNS was excited at 330 nm and its fluorescence viewed at 540 nm. The band pass of the excitation and emission monochromator slits was about 30 nm. The polarization, P, was calculated as $P = \frac{I_{vv} - GI_{vh}}{I_{vv} + GI_{vh}}$ where I_{vv} and I_{vh} are the fluorescence intensities with the analyzer vertical and horizontal and G is the grating correction factor, equal to 0.892 at 540 nm and 0.833 at 550 nm.

RESULTS

To facilitate discussion, the DNS-collagen conjugate will be designated DNC; the FI-collagen conjugate, FIC; and the pronase-treated conjugates, P-DNC and P-FIC.

The fluorescence polarization as a function of temperature for FIC and P-FIC is presented in Figure 1 as a Perrin plot of the reciprocal of P, the polari-

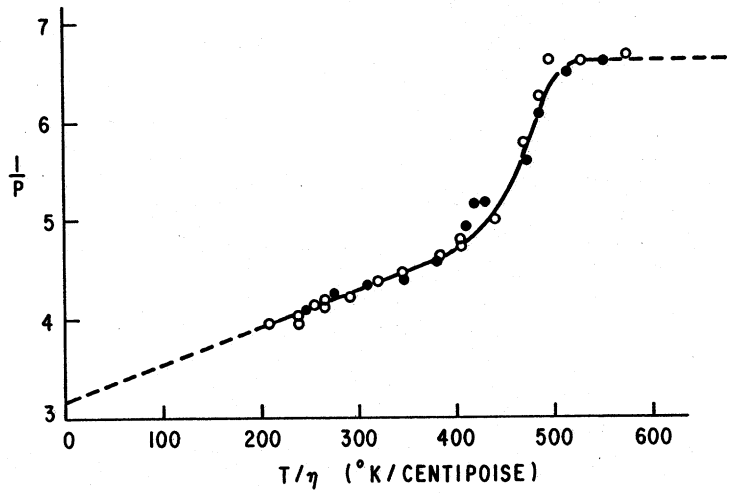

FIGURE 1.—Perrin plots for P-FIC and FIC in 0.10 M acetic acid. Concentration of protein-dye conjugate was 0.20 percent in both cases. (●) P-FIC; (O) FIC.

zation, *versus* T/η, the ratio of the absolute temperature to the viscosity of pure water. Both sets of points fall on the same curve. In the temperature range 35°–40°C., calfskin corium collagen undergoes a thermal transition (2), an unwinding of the triple helix. The unwound peptide chains have greater flexibility, and thus the rotational freedom of dye molecules bound to these chains should be greatly enhanced.

The curve shows a rather steep increase in the 35°–40°C. temperature range, indicating a considerable decrease in polarization as expected. Perrin plots of polarization data for DNC and P-DNC are shown in Figure 2. The plot for DNC is similar in contour to that for FIC and P-FIC. It shows considerable

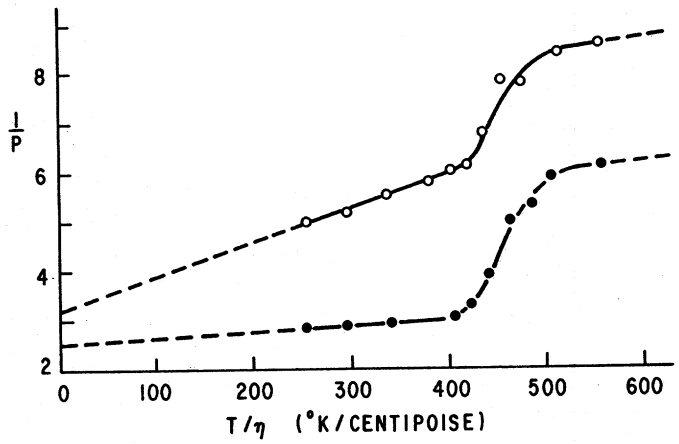

FIGURE 2.—Perrin plots for P-DNC and DNC in 0.10 M acetic acid. Concentration of protein-dye conjugate was 0.07 percent in both cases. (●) P-DNC; (O) DNC.

depolarization in the temperature range 10°–35°C. and then a rather steep increase in the transition temperature range. The Perrin plot for P-DNC is quite different. Very little depolarization occurs prior to the transition temperature, and it is interesting to note that even above the transition range the rotational freedom of dye molecules bound to P-DNC is less than that of at least some of the dye molecules bound to DNC.

The stability of collagen in solution is dependent on the pH, ionic strength, and type of ion present. The effect of these solution variables on the fluorescence polarization of DNC and P-DNC was studied. Figure 3 shows the effect of pH on the fluorescence polarization of DNC and P-DNC in 0.02 M $CaCl_2$. The pH of the solutions was adjusted by adding small amounts of dilute KOH or dilute HCl. The fluorescence polarization of DNC remains unchanged over the entire pH range from 2.5 to 10.5. The fluorescence polarization of P-DNC undergoes only a slight decrease as the pH increases from 3 to about 7.5 but

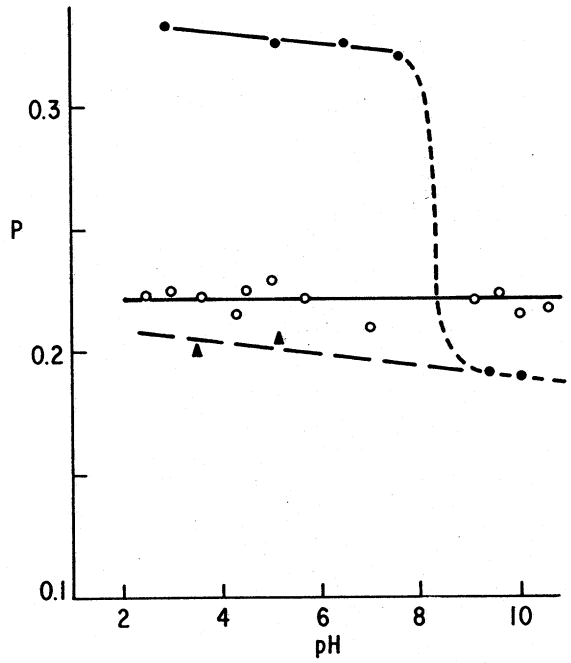

FIGURE 3.—Polarization of fluorescence of P-DNC and DNC as a function of pH in 0.02 M $CaCl_2$ at 25°C. Concentration of protein-dye conjugate was 0.07 percent. (●) P-DNC; (O) DNC; (▲) P-DNC acidified from pH 10.

is dramatically lower at more alkaline pH values. Moreover, when a P-DNC solution at pH 10 is acidified to lower pH, the fluorescence polarization does not recover, indicating an irreversible transition has taken place.

Ions such as Ca^{2+}, Mg^{2+}, SCN^-, and several others are known to reduce the thermal stability of collagen (1), that is, to lower its thermal transition temperature. Ions such as Na^+, K^+, and Cl^- do not produce this effect. Figures 4 and 5 show the effects of the salts KCl and $CaCl_2$ on the fluorescence polarization of DNC and P-DNC. KCl has no effect on the fluorescence polarization of either P-DNC or DNC up to the highest concentrations achievable without precipitation of collagen. Ca^{2+} ion has a solubilizing effect on collagen, and much higher concentrations could be achieved without collagen precipitating. As Figure 5 shows, the fluorescence polarization of both DNC and P-DNC is affected by $CaCl_2$. P-DNC appears to be more sensitive to the presence of $CaCl_2$, since at $CaCl_2$ concentrations greater than 0.4 M its fluorescence is depolarized to a greater extent than that of DNC.

DISCUSSION

For fluorescent dye molecules that are rigidly attached to proteins, the mean harmonic rotational relaxation time of the protein, ρ_h, and the lifetime of the

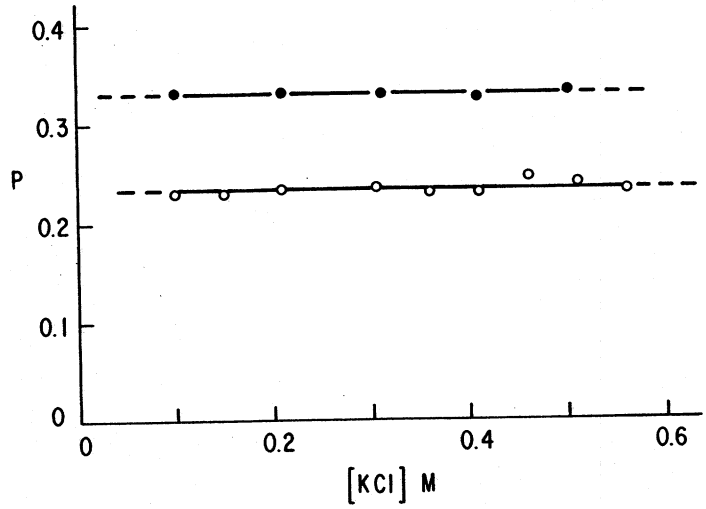

FIGURE 4.—Polarization of fluorescence from P-DNC and DNC as a function of [KCl] in 0.10 M acetic acid, pH 2.9. Concentration of protein-dye conjugate was 0.07 percent. Temperature 25°C. (●) P-DNC; (O) DNC.

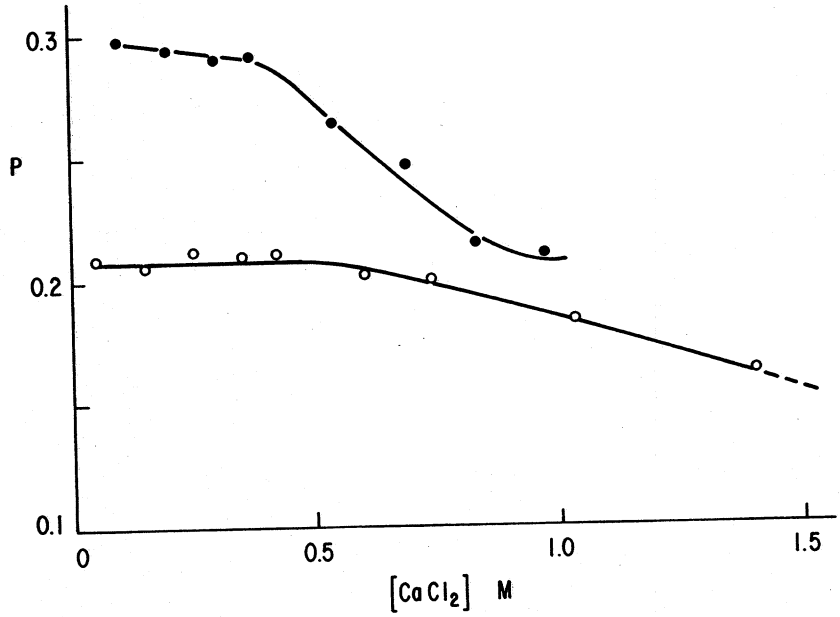

FIGURE 5.—Polarization of fluorescence from P-DNC and DNC as a function of [$CaCl_2$] in 0.05 M acetic acid-acetate, pH 5.0. Concentration of protein-dye conjugate was 0.05 percent. Temperature 25°C. (●) P-DNC; (O) DNC.

excited state of the dye, τ, are related by the modified Perrin-Levshin equation (9):

$$[(1/P) - (1/3)] = [(1/P_o) - (1/3)] [1 + (3\tau/\rho_h)] \quad . \quad (1)$$

Since ρ_h is proportional to η/T, a plot of $1/P$ *vs.* T/η would be linear. If $\rho_h > \tau$, then P would never differ very much from P_o, the value of P at infinite viscosity, and the Perrin plot would be almost a horizontal line. ρ_h for collagen is much larger than the excited state lifetimes of FI and DNS (8), and, if these dyes were rigidly bound to collagen, their fluorescence should undergo only a slight depolarization. The Perrin plot for P-DNC (Figure 2) most closely approaches this situation. The DNS molecules of P-DNC must be quite firmly held in a relatively rigid system.

The fluorescence of a bound dye can be depolarized by processes other than rotational diffusion of the entire protein molecule (9). If the section of the protein to which the dye is bound is flexible, that is, can undergo some movement with respect to the rest of the protein, then this local motion can depolarize the dye fluorescence. It is also possible, in some cases, for the dye to rotate about the covalent bond joining it to the protein. This "bond rotational diffusion" will also depolarize dye fluorescence. This latter type of process probably accounts for the depolarization of FIC and P-FIC fluorescence in the temperature range 10° to 35°C., shown in Figure 1. We arrive at this conclusion indirectly. The fluorescence of P-FIC is certainly not depolarized by rotational diffusion of the collagen molecule as a whole. The horizontal Perrin plot of P-DNC indicates there is very little local flexibiilty in the helical regions, and thus the fluorescence from P-FIC is most probably depolarized by rotation of the dye molecules about the covalent bonds linking them to the protein.

DNC and P-DNC present a different situation. The DNS molecules bound to collagen can be separated into two classes: those bound to helical regions and those bound to nonhelical regions. In P-DNC all the DNS molecules are bound to helical regions. Since the fluorescence of DNS molecules bound to helical regions is largely free of depolarization, the increased depolarization of fluorescence shown by DNC must be due to local diffusional motions in the nonhelical regions. Thus the difference in fluorescence polarization between DNC and P-DNC under a set of identical solution conditions is a measure of flexibility in the nonhelical regions.

The degree to which the fluorescence from DNC is depolarized does not depend upon the pH of the solution over the range 2.5 to 10.5 (Figure 3). This implies that the motional freedom of the fluorescing dye molecules, and presumably the protein structures to which they are bound, remains constant over that pH range. As mentioned before, fluorescence from the dye molecules bound to the helical regions of collagen is only slightly depolarized because of the structural rigidity of these regions. It would not be greatly surprising if the rigidity

of these helical regions was maintained over a wide pH range since the native helical conformation is stable up to at least pH 9.5 (10). On the other hand, the partial depolarization of fluorescence from DNC is due to dye molecules bound to the nonhelical regions, and it is rather surprising that the degree of motional freedom (that is, the local flexibility of these regions) remains the same over the entire pH range 2.5 to 10.5. Figures 4 and 5 show the fluorescence polarization of DNC in acidic solution also is unaffected by ionic strength changes from about 0.1 to 1.5 M (0.5 M $CaCl_2$). The fact that the motional freedom, or local flexibility in the nonhelical region, is insensitive to these rather drastic changes in solution environment suggests an aggregated or associated structure for the peptide chains in this region rather than unassociated peptide chains in random diffusional motion in the solvent medium. It is already known that in some collagen molecules the telopeptides are at least partially associated, owing to intramolecular crosslinks (5). It may be that further interaction of a non-bonded nature takes place to form an associated aggregate with a moderate degree of structural rigidity.

As can be seen in Figure 5, at $CaCl_2$ concentrations greater than 0.5 M, the fluorescence polarization of DNC is lowered. Ca^{2+} ions are known to decrease the thermal stability of the collagen triple helix (11). Thus, the decrease in polarization might be due to structural changes in either the helical or non-helical regions. In P-DNC molecules the nonhelical peptides are absent, and the decrease in polarization of P-DNC with increasing $CaCl_2$ concentration (Figure 5) might very well be due to structural changes that lead to local flexibility in the helical regions of collagen. It is interestitng that the decrease in fluorescence polarization of P-DNC does not occur precipitiously, which would indicate a complete unwinding of the helices above a critical Ca^{2+} ion concentration. The decrease occurs gradually over the concentration range of 0.4 M to 0.85 M $CaCl_2$, a fact indicating a progressive weakening of the helical structure leading to eventual complete unwinding as the concentration of $CaCl_2$ exceeds 0.85 M. The fluorescence polarization of DNC similarly decreases gradually as the $CaCl_2$ concentration increases. The decrease is more gradual than that of P-DNC and commences at a slightly higher $CaCl_2$ concentration, which tends to indicate that the helical structure of P-DNC is more sensitive to Ca^{2+} ion perturbation than that of DNC. This increased sensitivity is presumably due to the absence of the peptide segments in the nonhelical region.

The results shown in Figure 3 also indicate a difference in the stabilities of the helical regions of P-DNC and DNC in the alkaline pH region. At alkaline pH the fluorescence polarization of P-DNC falls to values close to those it has above the thermal transition temperature (see Figure 2). Also, the degree of polarization does not recover with acidification from alkaline pH. This indicates that the helical structure of P-DNC has been lost, while that of DNC remained intact under the same conditions of pH and temperature. These results

are in contrast to those of Hayashi and Nagai (4) who recently studied the thermal stability of pepsin-treated and untreated calfskin collagen in acidic and neutral solution. They found the thermal transition temperatures of the treated and untreated collagens to be almost identical. From this they concluded that the nonhelical regions of collagen do not contribute to the thermal stability of the protein. Our results indicate this conclusion may not be valid in alkaline solution.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the instrumentation skills of Henry J. Scherr, who assisted in the assembly of the fluorescence polarization spectrometer, and the in-depth knowledge of collagen structure and stimulating discussions that Leo D. Kahn provided during the period of this research.

REFERENCES

1. Weber, G. *Biochem. J.*, **51**, 145 (1952).
2. Ramachandran, G. N. "Treatise on Collagen," Vol. I, Academic Press, New York, 1967.
3. Yuan, L., and Veis, A. *Biopolymers*, **12**, 1437 (1973).
4. Hayashi, T., and Nagai, Y. *J. Biochem. (Tokyo)*, **73**, 999 (1973).
5. Davison, P. F., and Drake, M. P. *Biochemistry*, **5**, 313 (1966).
6. Kahn, L. D., and Witnauer, L. P. *J. Appl. Polym. Sci.*, **13**, 141 (1969).
7. Chen, R. F., and Bowman, R. L. *Science*, **147**, 729 (1965).
8. Steiner, R. F., and McAlister, A. J. *J. Polym. Sci.*, **24**, 105 (1957).
9. Weber, G. *Advances in Protein Chem.*, **8**, 415 (1953).
10. Bensusan, H. B., Mumaw, V. R., and Scanu, A. W. *Biochemistry*, **1**, 215 (1962).
11. Von Hippel, P. H., and Wong, K. Y. *Biochemistry*, **2**, 1399 (1963).